DOI:
10.1159/000487159

Document Version
Publisher's PDF, also known as Version of record

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:656-666
DOI: 10.1159/000487159
Published online: February 05, 2018
Accepted: December 12, 2017

Original Paper

Identifying Signalling Pathways Regulated by GPRC5B in β-Cells by CRISPR-Cas9-Mediated Genome Editing

Patricio Atanes Inmaculada Ruz-Maldonado Ross Hawkes Bo Liu
Shanta J. Persaud Stefan Amisten

Department of Diabetes, Faculty of Life Sciences & Medicine, Hodgkin Building, King's College London, Guy's Campus, London, UK

Key Words
β-cell • GPRC5B • CRISPR-Cas9 • Apoptosis • Proliferation • Type 2 diabetes

Abstract
Background/Aims: CRISPR-Cas9, a RNA-guided targeted genome editing tool, has revolutionized genetic engineering by offering the ability to precisely modify DNA. GPRC5B is an orphan receptor belonging to the group C family of G protein-coupled receptors (GPCRs). In this study, we analysed the functional roles of the Gprc5b receptor in MIN6 β-cells using CRISPR-Cas9 and transient over-expression of Gprc5b. ***Methods:*** The optimal transfection reagent for use in MIN6 β-cells was determined by analysing efficiency of GFP plasmid delivery by cell sorting. A MIN6 β-cell line in which Gprc5b expression was knocked down (Gprc5b KD) was generated using CRISPR-Cas9 technology. Gprc5b receptor mRNA expression, proliferation, apoptosis, Cignal 45-Pathway Reporter Array signalling and western blot assays were carried out using Gpcr5b KD MIN6 β-cells that had been transiently transfected with different concentrations of mouse Gprc5b plasmid to over-express Gprc5b. ***Results:*** JetPRIME® was the best candidate for MIN6 β-cell transfection, providing approximately 30% transfection efficiency. CRISPR-Cas9 technology targeting Gprc5b led to stable knock-down of this receptor in MIN6 β-cells and its re-expression induced proliferation and potentiated cytokine- and palmitate-induced apoptosis. The Cignal 45 Reporter analysis indicated Gprc5b-dependent regulation of apoptotic and proliferative pathways, and western blotting confirmed activation of signalling via TGF-β and IFNγ. ***Conclusion:*** This study provides evidence of CRISPR-Cas9 technology being used to down-regulate Gprc5b expression in MIN6 β-cells. This strategy allowed us to identify signalling pathways linking GPRC5B receptor expression to β-cell proliferation and apoptosis.

S. Persaud and S. Amisten contributed equally to this work.

Patricio Atanes
and Shanta J. Persaud

Department of Diabetes, Faculty of Life Sciences & Medicine,
Hodgkin Building, King's College London, Guy's Campus, London (UK)
Tel. +44 20 7848 6282, E-Mail shanta.persaud@kcl.ac.uk, patricio.atanes_juiz@kcl.ac.uk

Introduction

The simplicity of the CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9) system has revolutionized genome engineering. Initially described as an adaptive immune system in bacteria, it has been extensively developed as an RNA-guided genome editing tool, allowing precise manipulation of specific genomic loci and facilitating detailed investigation of targeted gene functions or diseases. CRISPR-Cas9 technology has been used for genome editing in induced pluripotent stem cells [1, 2] primary human endothelial cells [3] and the INS-1 [4, 5] and MIN6 [6, 7] β-cell lines, among others.

G protein-coupled receptors (GPCRs) constitute the largest family of transmembrane receptors encoded by the human genome [8], being targeted for the treatment of diseases such as diabetes and its associated complications [9]. Orphan GPCRs, whose endogenous ligand has not yet been identified, exhibit an appealing source of undiscovered therapeutic potential. GPRC5B, also known as retinoic acid-inducible gene 2 (Raig2), is a member of the Raig subfamily of type 3 (family C) GPCRs [10]. This orphan receptor is highly expressed in human and mouse islets [11], brain [12] and white adipose tissue [13]. Observations that it is up-regulated in islets from donors with type 2 diabetes (T2D) [11] and that its deletion in mice causes glucose intolerance [13] suggest that it may be involved in the pathogenesis of diabetes, but the molecular mechanisms underlying this remain unclear.

As there are no known specific agonists for Gprc5b we have previously used lentivirus to down-regulate Gprc5b in islets and we demonstrated that this confers protection against apoptosis [11]. In the current study, we have generated isogenic MIN6 β-cell clones in which Gprc5b has been specifically and stably knocked down using the CRISPR-Cas9 technology. We have then reintroduced Gprc5b in a graded fashion to determine the effects on β-cell proliferation and apoptosis, and the cell signalling pathways downstream of Gprc5b in β-cells.

Materials and Methods

Reagents

FuGENE® 6 Transfection Reagent, Dual-Glo® Luciferase Assay System kits, anti-mouse and anti-rabbit IgG (H+L), HRP-conjugated secondary antibodies and caspase 3/7 assay kits were from Promega UK (Southampton, UK). The mouse Gprc5b antibody was from Biorbyt (Cambridge, UK). Linear polyethylenimine was from Polysciences, Inc. (Hirschberg an der Bergstrasse, Germany), Metafectene® Pro from Cambio (Cambridge, UK), jetPRIME® from Polyplus-transfection (Illkirch, France), Torpedo^{DNA} transfection reagent from Thistle Scientific Ltd. (Glasgow, UK) and Lipofectamine® 2000 Transfection Reagent and Pierce™ BCA Protein Assay Kit were from Thermo Fisher Scientific (Loughborough, UK). NanoJuice® Transfection Kit was from Millipore (U.K.) Ltd. (Hertfordshire, UK). Attractene Transfection Reagent, DNeasy Blood & Tissue kits and Cignal 45-Pathway Reporter Array kits were from Qiagen Ltd. (Manchester, UK). Recombinant murine TNFα, IFNγ and IL-1β were from PeproTech EC Ltd. (London, UK). The BrdU cell proliferation kit was from Sigma-Aldrich (Dorset, UK). Gprc5b double nickase plasmid (mouse) and anti-β-actin antibody were from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Anti-Smad2/3, anti-STAT1, anti-Smad2/3 (phospho T8) and anti-STAT1 (phospho S727) antibodies were from Abcam plc. (Cambridge, UK). All other chemicals were from Sigma Aldrich or Thermo Fisher Scientific.

Cell culture

MIN6 β-cells (passage 25-45), were maintained in culture in a humidified incubator at 37°C, 5% CO_2, 95% air in DMEM supplemented with 25 mM glucose, 2 mM glutamine, 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 100 μM 2-mercaptoethanol. The Gpcr5b KD MIN6 β-cell line was cultured with the same medium, which was also supplemented with 1.5 μg/ml puromycin as the resistance selection antibiotic.

Screening of MIN6 β-cell transfection efficiency

Approximately 200,000 MIN6 β-cells were seeded into 12-well cell culture plates and maintained in culture overnight, then transfected with a plasmid encoding GFP using a range of commercially available transfection reagents (FuGENE® 6, Linear polyethylenimine, Metafectene® Pro, jetPRIME®, Torpedo^{DNA}, Lipofectamine® 2000, NanoJuice® and Attractene) following the manufacturers' instructions. Cells were incubated for 48 hours after transfection, retrieved by trypsinisation then resuspended in 1 ml of sterile PBS. GFP production by transfected cells was analysed using a cell sorter that excited GFP fluorescence with a blue laser at 488nm (BD FACSAria III, Flow Cytometry Facilities, Guy's Hospital, London), and the expression was plotted versus fluorescence of untransfected MIN6 β-cells (GFP negative).

Gprc5b receptor knockout in MIN6 β-cells using CRISPR-Cas9 technology

Based on previous reports of a link between GPRC5B and T2D, we generated a stable Gpcr5b KD MIN6 β-cell line using CRISPR-Cas9 technology to investigate the direct role of this receptor in β-cell function. For this purpose, approximately 500,000 MIN6 β-cells were seeded into 6-well cell culture plates, maintained in culture overnight and transfected using jetPRIME® with 1 μg of a Gprc5b double nickase plasmid set that consists of a pair of plasmids, each encoding a D10A mutated Cas9 nuclease and a unique, target-specific 20 nt guide RNA (gRNA). Each pair of gRNA sequences are offset by approximately 20 bp to allow for high specificity gene knockout. The specific regions of the Gprc5b gene targeted by these plasmids are GCTCTTCGTGATCACCTCGG and GAAAACGCCAGCACATCCCG. This strategy was employed to induce Gpcr5b-targeted double strand breaks in MIN6 β-cells at the genomic locus at chromosome 7 (region F2, exon 4). Cells were maintained in culture for 48 hours at 37°C post-transfection then transferred into 96 well plates using a cell dilution method to allow isolation of single colonies (genome-targeted monoclonal cell clones). The culture medium was supplemented with 1.5 μg/ml puromycin to allow selection of cells expressing the transfected plasmids. After growing and generating stocks, the isogenic colonies were expanded, genomic DNA was extracted from each clone and expression of the CRISPR Gprc5b knockout sequence flanking the deleted region was confirmed by PCR using primers (*forward*: 5'-CTGAGGAAAGGAGCACGT-3' and *reverse*: 5'-CACAGGGCTCTTCTTCTCCTT-3') and subsequent Sanger sequencing (Source Bioscience).

RNA extraction and quantitative real-time PCR

Groups of approximately 200,000 Gpcr5b KD MIN6 β-cells were seeded into 6-well plates, maintained in culture overnight and transfected with increasing concentrations of mouse Gprc5b plasmid (0.01, 0.1, 1, 10 and 100%, bulked to 100% DNA with pcDNA3 vector to ensure the same amount of DNA per condition tested) using jetPRIME®. Un-transfected WT MIN6 β-cells were used as controls. After 48 hours total RNA was extracted using a modified TRIzol protocol [14] and Gprc5b, Nodal and DP5 (Hrk) gene expression relative to the house-keeping gene GAPDH was determined using QuantiTect qPCR assays, as described elsewhere [15, 16]. All gene primer efficiency (E) [17] values were in the range of 1.85–2.15. For all gene quantifications, template cDNAs were diluted such that all quantified genes returned Ct values <30.

Proliferation

Groups of 10,000 Gpcr5b KD MIN6 β-cells were seeded into 96-well plates and transfected the following day with increasing concentrations of mouse Gprc5b plasmid (0.01, 0.1, 1, 10 and 100%, bulked to 100% DNA with pcDNA3) using jetPRIME®. Un-transfected WT MIN6 β-cells were used as controls. After overnight incubation, cells were maintained for a further 24 hours in serum-free medium before being labelled with 100μM BrdU for 4h at 37°C. BrdU incorporation into proliferating β-cells was quantified using a plate reader measuring absorbance at 450 nm [18].

Apoptosis

Groups of 10,000 Gpcr5b KD MIN6 β-cells were seeded into 96-well white opaque cell culture plates and transfected the following day with increasing concentrations of mouse Gprc5b plasmid (0.01, 0.1, 1, 10 and 100%, bulked to 100% DNA with pcDNA3) using jetPRIME®. Un-transfected WT MIN6 β-cells were used as controls. Quantification of basal, cytokine and palmitate-induced β-cell apoptosis was carried out using the Caspase-Glo 3/7 assay kit, essentially as previously described [18].

Cignal™ Finder 45-Pathway Reporter Array

Pathway reporters were introduced into Gpcr5b KD MIN6 β-cells in 96-well cell culture plates via reverse transfection according to the manufacturer's protocol. Briefly, reporter DNA constructs resident in each plate well were resuspended with 50 µl Opti-MEM, together with pcDNA3 (100%) or mouse Gprc5b plasmid (10%, bulked with pcDNA3 to 100%) and then mixed with jetPRIME®. Cells were suspended in Opti-MEM supplemented with 10% of fetal bovine serum and 0.1 mM non-essential amino acids, and then 50 µl of the cell suspension, equivalent to 10,000 cells, was added into each plate well and mixed with DNA resident in the plate, plasmids and added transfection reagent. The cells were incubated for 48 h at 5% CO_2 and 37 °C. Analysis of 45 signalling pathways in the cells was then carried out using the Cignal 45-Pathway Reporter Array kit by quantifying the luminescent signal according to the manufacturer's instructions. Expression values were normalised by establishing a sample ratio value to discriminate between genuine cellular responses (Firefly luminescent signal) and non-specific responses (Renilla luminescent signal), and plotted as log2 fold change comparing cells expressing 10% mouse Gprc5b plasmid with those expressing the pcDNA3 control plasmid.

Western Blot

Gprc5b expression in wildtype MIN6 cells and those transfected with the Gprc5b double nickase plasmid set was determined by western blotting using an anti-Gprc5b antibody at a dilution of 1/100 and normalised to beta-actin expression in the same samples (1/2,000 dilution). For identification of signalling downstream of Gprc5b in MIN6 β-cells, groups of approximately $4x10^6$ Gpcr5b KD MIN6 β-cells were seeded into T75 flasks, maintained in culture overnight and transfected with 10% mouse Gprc5b plasmid (plus 90% of pcDNA3 empty vector) or 100% of pcDNA3 using jetPRIME®. After 48 hours incubation, proteins were extracted, fractionated on 4-12% Bis-Tris gels (200V, 1 hour) then immunoprobed on PVDF membranes with antibodies directed against beta-actin (1/2,000), total (1/200) or phosphorylated (1/200) anti-Smad2/3 or total (1/500) or phosphorylated (1/1,000) anti-STAT1 and their respective secondary antibodies.

Statistical analysis

GraphPad Prism 5.0 (GraphPad Software, Inc.) was used for statistical analyses. Data are presented as mean±SEM and analysed using ANalysis Of VAriance (ANOVA) or Student's t tests and $p<0.05$ was considered statistically significant.

Results

MIN6 β-cell line transfection efficiency screening

The MIN6 β-cell line is relatively difficult to transfect compared to some continuous cell lines such as HeLa and HEK-293T so eight commercially available transfection reagents were screened for their utility in delivering a GFP plasmid to MIN6 β-cells to drive GFP expression. Sorting of GFP fluorescent cells 48 hours post-transfection indicated that the jetPRIME® reagent in a 1:2 DNA to transfection reagent ratio (w/v) was the most effective of the eight compounds tested, with almost 30% of cells positive for GFP expression. All other reagents tested failed to reach these transfection efficiency levels, with only two other reagents (FuGENE® 6 and Torpedo^{DNA}) having transfection efficiencies in excess of 10%. jetPRIME® was therefore used for all subsequent transfection protocols described herein.

CRISPR-Cas9-mediated knock-down of MIN6 β-cell GPRC5B

CRISPR-Cas9 technology was used to induce Gpcr5b-targeted double strand breaks at the genomic locus in MIN6 β-cells. Individual puromycin-resistant transfected cells were expanded and screened for Gpcr5b-targeted double strand breaks using PCR and Sanger sequencing. A single stable Gpcr5b KD MIN6 β-cell line, in which the sequence homology was reduced to the first 29 amino acids of the 410 amino acids of full length Gprc5b, with the additional introduction of an early stop codon downstream (Fig. 1A), was used as a starting point for further experiments where Gprc5b was transiently re-introduced into the cells. Western blotting confirmed Gprc5b knockdown in these cells, with approximately 60%

reduction in Gprc5b protein expression in the KD cells compared to protein-matched WT MIN6 cells (Fig. 1B, C).

Graded re-introduction of Gprc5b into MIN6 β-cells

Gprc5b was re-introduced into the Gprc5b KD MIN6 β-cell line by transient transfection with increasing concentrations of mouse Gprc5b plasmid. It can be seen from Fig. 2 that CRISPR-Cas9-targeted deletion of Gprc5b resulted in significant reduction in Gprc5b mRNA levels compared to WT MIN6 β-cells, as expected. Delivery of 0.01-100% of the Gprc5b plasmid to Gprc5b KD cells resulted in a concentration-dependent increase in Gprc5b mRNA expression, such that cells transfected with 100% mouse Gprc5b plasmid had a 1,340-fold up-regulation of Gprc5b (Fig. 2).

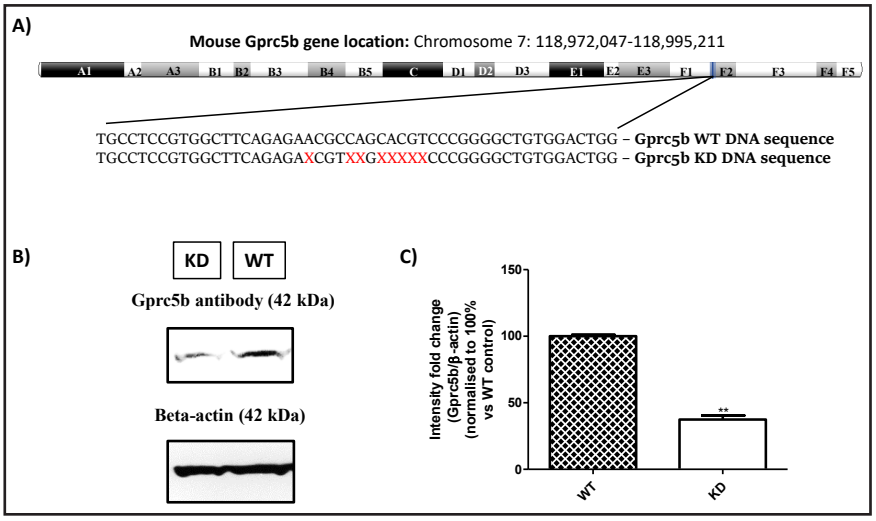

Fig. 1. Generating a Gpcr5b KD MIN6 β-cell line using CRISPR-Cas9 technology. A) Schematic representation of the DNA sequence targeted using CRISPR-Cas9 technology to generate a Gpcr5b KD MIN6 monoclonal cell clone, highlighting the deleted nucleotides in the KD sequence, leading to frame shift mutations and followed by several novel stop codons, truncating the protein expression. B) Western blotting validation of Gprc5b KD in a MIN6 β-cell clone, compared to protein-matched WT MIN6 cells. C) Densitometric analysis of GPRC5B protein expression in KD and WT MIN6 cells, normalised to beta-actin. Values are means + range; n=2. **p<0.01.

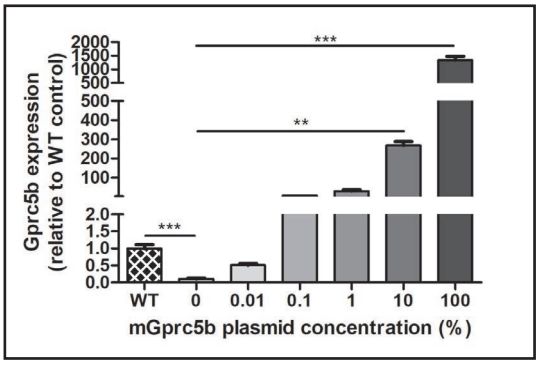

Fig. 2. Gprc5b mRNA expression in MIN6 β-cell lines. Gpcr5b KD MIN6 β-cells (0%) were transfected with increasing concentrations of mouse Gprc5b plasmid (0.01-100%) using jetPRIME®. Data are normalised relative to the reference gene Gapdh and presented as expression relative to WT MIN6 β-cells. Values are expressed as means + SEM; n=3. ***p<0.001; **p<0.01.

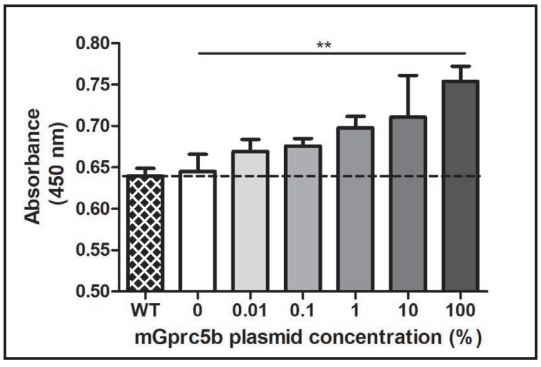

Fig. 3. Effect of Gprc5b expression on MIN6 β-cell proliferation. Gpcr5b KD MIN6 β-cells (0%) were transfected with increasing concentrations of mouse Gprc5b plasmid (0.01-100%) using jetPRIME®. Proliferation was determined by quantification of BrdU incorporation into newly synthesised DNA. Data are presented as absorbance values (450 nm). Values are expressed as means + SEM; n=6-8. **p<0.01.

Effect of Gprc5b expression on MIN6 β-cell proliferation

The role of Gprc5b in β-cell proliferation was investigated using Gpcr5b KD MIN6 β-cells transfected with increasing concentrations of mouse Gprc5b plasmid. There was no significant difference between the rate of proliferation of WT MIN6 β-cells and Gpcr5b KD MIN6 β-cells, but re-introduction of Gprc5b induced a concentration-dependent stimulation of BrdU incorporation into proliferating MIN6 cells, with maximal effects achieved in cells that had been transfected with 100% of the plasmid (Fig. 3).

Effect of Gprc5b expression on MIN6 β-cell apoptosis

It can be seen from Fig. 4A that deletion of Gprc5b did not affect basal apoptosis of MIN6 β-cells, but cells that had been transfected with 100% of the mouse Gprc5b plasmid showed a significant elevation in caspase 3/7 activities. Similar pro-apoptotic effects of over-expression of Gprc5b were observed under conditions where enhanced apoptosis was induced by a mixed cytokine cocktail (Fig. 4B) or by the saturated long chain fatty acid palmitate (Fig. 4C). Thus, exposure of Gpcr5b KD MIN6 β-cells for 20 hours to a cocktail of TNFα, IFNγ and IL-1β or to 500μM palmitate promoted 3-fold and 2-fold elevations in apoptosis, respectively, and this was further increased in a concentration-dependent manner by re-introduction of Gprc5b.

Signalling pathway screening by re-introduction of Gprc5b into MIN6 β-cells

Gprc5b is expressed by islets where it may play an important role in β-cell function [11], but the molecular signalling pathways downstream of Gprc5b in β-cells have not been previously identified. Transfection of Gpcr5b KD MIN6 β-cells with 10% mouse Gprc5b plasmid (bulked to 100% using empty pcDNA3) resulted in identification of several cascades that were specifically and consistently up-regulated (Fig. 5). Consistent with the MIN6 β-cell apoptosis data provided here, 3 of the top 4 array pathways (progesterone receptor, TGF-β and IFNγ) are reported to be pro-apoptotic in β-cells [19-21]. In addition, Gprc5b over-expression

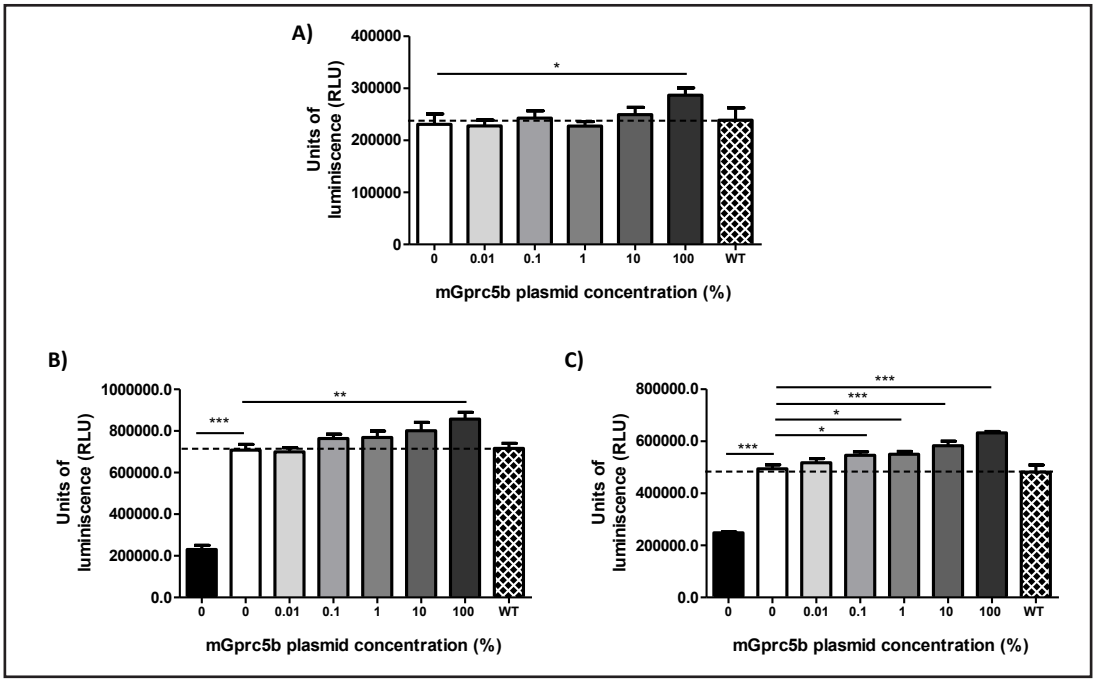

Fig. 4. Effect of Gprc5b expression on MIN6 β-cell apoptosis. Gpcr5b KD MIN6 β-cells (0%) were transfected with increasing concentrations of mouse Gprc5b plasmid (0.01-100%) using jetPRIME®. Basal (A), cytokine- (B) and palmitate- (C) induced apoptosis was quantified by measuring caspase 3/7 activities. Data are expressed as relative light units (RLU). Values are means + SEM; n=6. *p < 0.05; **p<0.01; ***p<0.001.

was associated with up-regulation of the pro-proliferative E2F transcription factor [22] and down-regulation of HN4α, a repressor of proliferation [23], in keeping with our observations of enhanced proliferation following increased Gprc5b expression in MIN6 β-cells.

Gprc5b regulates TGF-β and IFNγ signalling pathways in MIN6 β-cells.

Two key Gprc5b-regulated pathways identified by the Cignal Reporter Arrays, TGF-β and IFNγ, were further probed by western blotting of MIN6 β-cells using antibodies directed against SMAD2/3 and STAT1, proteins downstream of TGF-β and IFNγ signalling. Transfection of Gprc5b KD MIN6 β-cells with 10% mouse Gprc5b plasmid had no effect on total SMAD2/3 (Fig. 6A) or STAT1 (Fig. 6B) expression, but phosphorylated, active SMAD2/3 (Fig. 6A, C) and STAT1 (Fig. 6B, D) proteins were significantly higher following β-cell overexpression of Gprc5b. We also quantified expression of mRNAs encoding Nodal and DP5, which signal through SMAD2/3 [20] and STAT1[21] cascades respectively. Nodal and DP5 mRNAs were significantly reduced following CRISPR-mediated knock-down of Gprc5b expression, and cells that had been transfected with 10% Gprc5b plasmid showed substantial up-regulation of Nodal mRNA (Fig. 6E), while DP5 mRNA was not significantly elevated (Fig. 6F).

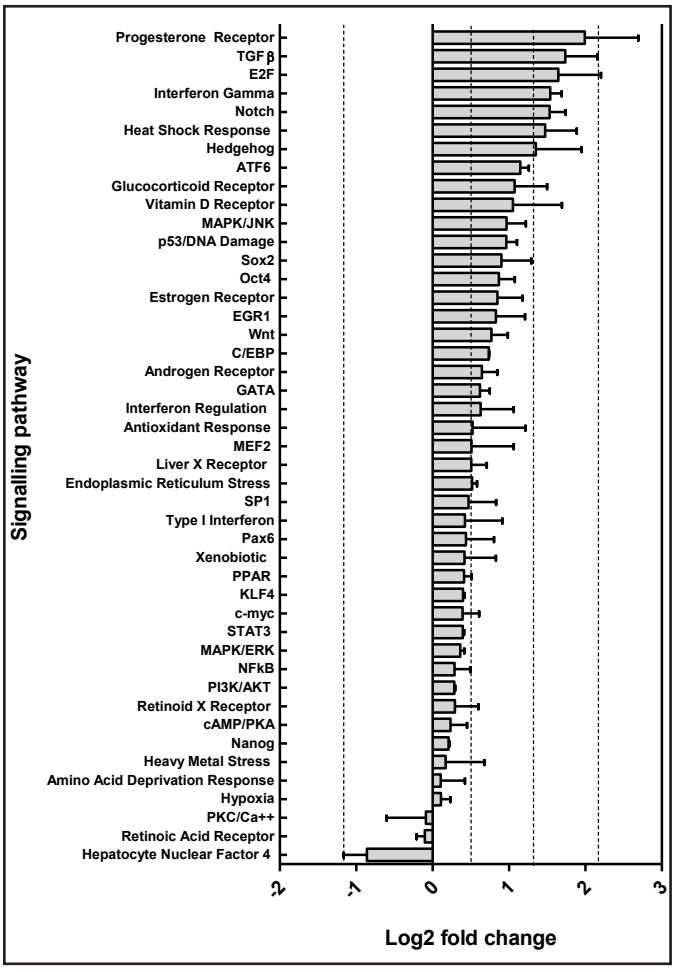

Fig. 5. Gprc5b-regulated signalling in MIN6 β-cells. Gprc5b KD MIN6 β-cells were transfected with 10% mouse Gprc5b plasmid or 100% pcDNA3 vector and with Cignal Array reporter constructs. Pathway reporters are listed by decreasing values of log2 ratio fold-changes in cells transfected with 10% mouse Gprc5b plasmid compared to cells transfected with pcDNA3. Data are expressed as means + range; n =2.

Discussion

Insulin-secreting cell lines represent an alternative platform to primary islet β-cells for studying biological and physiological processes, and they may be genetically manipulated by transfection to investigate the roles of specific genes. MIN6 β-cells retain many of the physiological characteristics of primary mouse β-cells, and they have been extensively used for functional studies [24, 25]. However, MIN6 β-cells are not particularly amenable to transfection, which has led to difficulties in modifying gene expression for functional analyses [26, 27]. In this study we identified that jetPRIME® was the most effective candidate for use with MIN6 β-cells, with a transfection efficiency of ~30% at 48 hours.

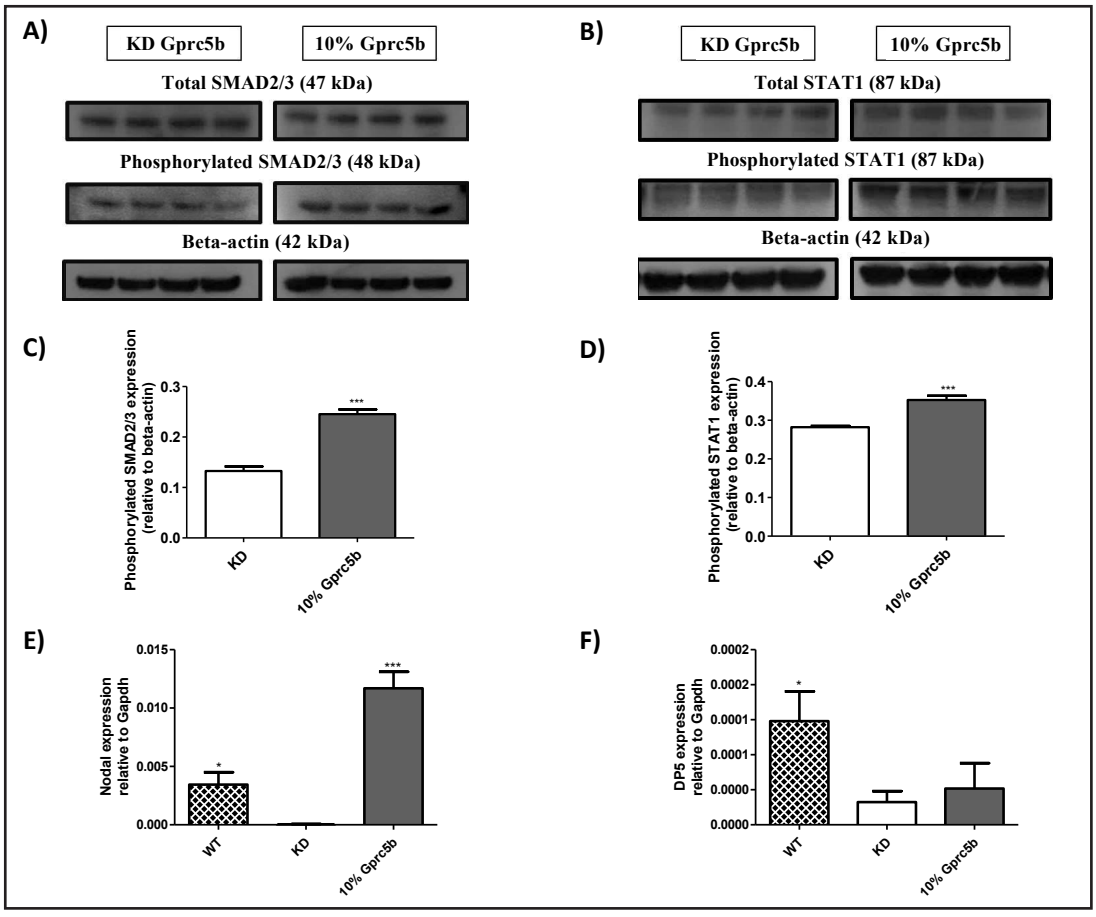

Fig. 6. Gpcr5b regulation of TGF-β and IFNγ signalling pathways in MIN6 β-cells. Gprc5b KD MIN6 β-cells were transfected with 100% empty pcDNA3 vector (KD) or with 10% mouse Gprc5b plasmid (10% Gprc5b) and harvested 48 hours post-transfection. (A, B) Fractionated proteins were immunoanalyzed for total and phosphorylated SMAD2/3 and STAT1 proteins, and compared to beta-actin expression in the same samples. (C, D) Densitometric analysis of phosphorylated SMAD2/3 (C) and STAT1 (D) normalised to beta-actin. Values are means + SEM; n=4. ***p<0.001. (E, F) Quantification of mRNAs encoding Nodal (E) and DP5 (F) in WT MIN6 β-cells and in Gprc5b KD MIN6 β-cells transfected with or without 10% Gprc5b plasmid. Data are normalized to Gapdh. Values are means + SEM; n = 5. *p < 0.05; ***p<0.001 relative to expression in KD cells.

GPCRs represent the largest superfamily and most diverse group of mammalian transmembrane proteins and over 30% of currently marketed drugs target human GPCRs [8, 28]. Orphan receptors, those whose endogenous ligand has not yet been identified, have huge potential in biomedical research and drug development, including in the regulation of islet function [29]. One of these orphan receptors, GPRC5B, is widely expressed in human and mouse islets [11, 30], and we have previously reported that Gprc5b down-regulation increases insulin secretion and promotes β-cell survival [11]. In addition, GPRC5B expression was up-regulated in human islets from type 2 diabetic donors, suggesting a possible association between this receptor and β-cell dysfunction in diabetes [11]. Moreover, Gprc5b has also been associated with diet-induced obesity and T2D [13], with confirmed high expression by white adipose tissue [31].

Since no endogenous ligands have been identified for GPRC5B, we have adopted the approach of stably down-regulating this receptor in β-cells and then increasing its expression through transient transfection. We developed a standardised protocol for producing modified isogenic stable MIN6 β-cell clones under-expressing Gprc5b using CRISPR-Cas9 technology, in which a non-specific CRISPR-associated endonuclease, directed by a guide RNA molecule

to a specific DNA sequence, snips DNA like a pair of molecular scissors [32, 33]. This approach causes permanent and irreversible insertions, deletions or frameshifts in the targeted sequence, in contrast to RNA interference methods that result in a temporary reduction in protein expression. The increased proliferation and apoptosis observed following over-expression of Gprc5b in MIN6 β-cells suggests that this receptor can signal constitutively, as has been observed for other GPCRs [34], or that the β-cells express an endogenous agonist for Gprc5b, which can have autocrine effects when the receptor is expressed at high levels.

High-performance cell-based assays allow screening of multiple signalling activities simultaneously to identify relevant pathways for further analysis [35]. Using the Cignal™ 45-Pathway Reporter Array platform and the Gprc5b KD MIN6 β-cell line, we identified that apoptosis and proliferation pathways were up-regulated after Gprc5b plasmid transfection, and the anti-proliferative HNF4α pathway was down-regulated. We then focused on the IFNγ-regulated transcription factor STAT1 and TGF-β-regulated transcription factor SMAD2/3 as these proteins have previously been reported to promote β-cell apoptosis [20, 21]. Up-regulation of the TGF-β and IFNγ pro-apoptotic signalling pathways following re-introduction of Gprc5b into Gprc5b KD MIN6 β-cells was confirmed by western blotting. We also identified that Gprc5b KD MIN6 β-cells had lower levels of mRNAs encoding the pro-apoptotic proteins Nodal and DP5, which have been implicated in induction of β-cell death [20, 21, 36]. The down-regulation of these mRNAs in the Gprc5b KD MIN6 β-cells was not associated with reduced basal levels of apoptosis, most likely because unstimulated apoptosis in MIN6 β-cells is very low and not readily amenable to further reduction. The increase in Nodal mRNA following over-expression of Gprc5b is consistent with Gprc5b promoting β-cell apoptosis through a Nodal/Smad2/3 pathway, as has been reported for INS-1 insulin-secreting cells [20]. The role of DPF5 is less clear, because although its regulation by Gprc5b is implicated by the reduction in its expression in Gprc5b KD MIN6 β-cells, receptor over-expression did not significantly up-regulate DP5 mRNA levels. Nonetheless, the array and western blotting data indicate that Gprc5b activates IFNγ/STAT1 signalling in β-cells and the reduction in DP5 following Gprc5b knock-down suggests a role for this pro-apoptotic protein downstream of Gprc5b.

In addition to increased signalling through pro-apoptotic pathways, we also identified that Gprc5b couples to signalling via E2F, which is known to promote β-cell proliferation [37, 38], and its down-regulation of HNF4α signalling is consistent with the reported role of this transcription factor as an inhibitor of proliferation [23] and the effects of an HNF4α antagonist to stimulate β-cell proliferation [39]. It is unusual for individual receptors to be coupled to pathways that induce both apoptosis and proliferation, as has been identified here for Gprc5b, but it is possible that enhanced proliferation is required to compensate for the potentially deleterious effects of activation of the pro-apoptotic cascades described above.

Conclusion

In summary, we have used the CRISPR-Cas9 technology to specifically reduce Gprc5b expression in MIN6 β-cells, and used this model for graded re-introduction of this receptor for functional analyses. We have confirmed that Gprc5b promotes β-cell apoptosis, most likely through activation of the TGFβ/Nodal/SMAD2/3 and IFNγ/STAT1/DP5 signalling pathways, making it an appealing target for development of inhibitors to treat T2D.

Acknowledgements

The study was designed by SA, PA and SJP. Data were collected and analysed by PA, IRM, RH and BL. The article was drafted by PA, SA and SJP. All authors revised the article critically for intellectual content and gave their approval of this version to be published. PA, SJP and

SA take responsibility for the contents of the article. This study was supported by grants from the EFSD/Boehringer-Ingelheim Research Programme and a Diabetes UK RD Lawrence Fellowship to SA (11/0004172). All authors declare that they have no competing interests.

Disclosure Statement

The authors have nothing to disclose.

References

1 Kim EJ, Kang KH, Ju JH: CRISPR-Cas9: a promising tool for gene editing on induced pluripotent stem cells. Korean J Intern Med 2017;32:42-61.
2 Ding Q, Regan SN, Xia Y, Oostrom LA, Cowan CA, Musunuru K: Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. Cell Stem Cell 2013;12:393-394.
3 Abrahimi P, Chang WG, Kluger MS, Qyang Y, Tellides G, Saltzman WM, Pober JS: Efficient gene disruption in cultured primary human endothelial cells by CRISPR/Cas9. Circ Res 2015;117:121-128.
4 Naylor J, Suckow AT, Seth A, Baker DJ, Sermadiras I, Ravn P, Howes R, Li J, Snaith MR, Coghlan MP, Hornigold DC: Use of CRISPR/Cas9-engineered INS-1 pancreatic beta cells to define the pharmacology of dual GIPR/GLP-1R agonists. Biochem J 2016;473:2881-2891.
5 Bompada P, Atac D, Luan C, Andersson R, Omella JD, Laakso EO, Wright J, Groop L, De Marinis Y: Histone acetylation of glucose-induced thioredoxin-interacting protein gene expression in pancreatic islets. Int J Biochem Cell Biol 2016;81:82-91.
6 Wang Q, Pronin AN, Levay K, Almaca J, Fornoni A, Caicedo A, Slepak VZ: Regulator of G protein signaling Gbeta5-R7 is a crucial activator of muscarinic M3 receptor-stimulated insulin secretion. FASEB J 2017;10.1096/fj.201700197RR
7 Basiri M, Behmanesh M, Tahamtani Y, Khalooghi K, Moradmand A, Baharvand H: The Convenience of Single Homology Arm Donor DNA and CRISPR/Cas9-Nickase for Targeted Insertion of Long DNA Fragment. Cell J 2017;18:532-539.
8 Stockert JA, Devi LA: Advancements in therapeutically targeting orphan GPCRs. Front Pharmacol 2015;6:100.
9 Persaud S: Islet G-protein coupled receptors: therapeutic potential for diabetes. Current Opinion in Pharmacology 2017;37:24-28.
10 Chung HJ, Kim JD, Kim KH, Jeong NY: G protein-coupled receptor, family C, group 5 (GPRC5B) downregulation in spinal cord neurons is involved in neuropathic pain. Korean J Anesthesiol 2014;66:230-236.
11 Soni A, Amisten S, Rorsman P, Salehi A: GPRC5B a putative glutamate-receptor candidate is negative modulator of insulin secretion. Biochem Biophys Res Commun 2013;441:643-648.
12 Robbins MJ, Charles KJ, Harrison DC, Pangalos MN: Localisation of the GPRC5B receptor in the rat brain and spinal cord. Brain Res Mol Brain Res 2002;106:136-144.
13 Kim YJ, Sano T, Nabetani T, Asano Y, Hirabayashi Y: GPRC5B activates obesity-associated inflammatory signaling in adipocytes. Sci Signal 2012;5:ra85.
14 Amisten S, Braun OO, Bengtsson A, Erlinge D: Gene expression profiling for the identification of G-protein coupled receptors in human platelets. Thromb Res 2008;122:47-57.
15 Amisten S: Quantification of the mRNA expression of G protein-coupled receptors in human adipose tissue. Methods Cell Biol 2016;132:73-105.
16 Amisten S: A rapid and efficient platelet purification protocol for platelet gene expression studies. Methods Mol Biol 2012;788:155-172.
17 Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
18 Liu B, Barbosa-Sampaio H, Jones PM, Persaud SJ, Muller DS: The CaMK4/CREB/IRS-2 cascade stimulates proliferation and inhibits apoptosis of beta-cells. PLoS One 2012;7:e45711.

19 Zhou R, Yao X, Xu X, Wang G, Zhu Z, Chen J, Chen L, Shen X: Blockage of progesterone receptor effectively protects pancreatic islet beta cell viability. Steroids 2013;78:987-995.
20 Zhao F, Huang F, Tang M, Li X, Zhang N, Amfilochiadis A, Li Y, Hu R, Jin T, Peng C, Wang Q: Nodal induces apoptosis through activation of the ALK7 signaling pathway in pancreatic INS-1 beta-cells. Am J Physiol Endocrinol Metab 2012;303:E132-143.
21 Moore F, Naamane N, Colli ML, Bouckenooghe T, Ortis F, Gurzov EN, Igoillo-Esteve M, Mathieu C, Bontempi G, Thykjaer T, Orntoft TF, Eizirik DL: STAT1 is a master regulator of pancreatic {beta}-cell apoptosis and islet inflammation. J Biol Chem 2011;286:929-941.
22 Duronio RJ, Xiong Y: Signaling pathways that control cell proliferation. Cold Spring Harb Perspect Biol 2013;5:a008904.
23 Walesky C, Apte U: Role of hepatocyte nuclear factor 4alpha (HNF4alpha) in cell proliferation and cancer. Gene Expr 2015;16:101-108.
24 Ishihara H, Asano T, Tsukuda K, Katagiri H, Inukai K, Anai M, Kikuchi M, Yazaki Y, Miyazaki JI, Oka Y: Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. Diabetologia 1993;36:1139-1145.
25 Persaud SJ, Hauge-Evans AC, Jones PM: Chapter 15 - Insulin-Secreting Cell Lines: Potential for Research and Diabetes Therapy A2 - Ulloa-Aguirre, Alfredo; in (Conn PM, ed) Cellular Endocrinology in Health and Disease. Boston, Academic Press, 2014, vol. https://doi.org/10.1016/B978-0-12-408134-5.00015-9p.^pp. 239-256.
26 Harbeck MC, Chepurny O, Nikolaev VO, Lohse MJ, Holz GG, Roe MW: Simultaneous optical measurements of cytosolic Ca2+ and cAMP in single cells. Sci STKE 2006;2006:pl6.
27 Lai E, Bikopoulos G, Wheeler MB, Rozakis-Adcock M, Volchuk A: Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic beta-cells. Am J Physiol Endocrinol Metab 2008;294:E540-550.
28 Rask-Andersen M, Almen MS, Schioth HB: Trends in the exploitation of novel drug targets. Nat Rev Drug Discov 2011;10:579-590.
29 Ngo T, Kufareva I, Coleman J, Graham RM, Abagyan R, Smith NJ: Identifying ligands at orphan GPCRs: current status using structure-based approaches. Br J Pharmacol 2016;173:2934-2951.
30 Amisten S, Salehi A, Rorsman P, Jones PM, Persaud SJ: An atlas and functional analysis of G-protein coupled receptors in human islets of Langerhans. Pharmacol Ther 2013;139:359-391.
31 Robbins MJ, Michalovich D, Hill J, Calver AR, Medhurst AD, Gloger I, Sims M, Middlemiss DN, Pangalos MN: Molecular cloning and characterization of two novel retinoic acid-inducible orphan G-protein-coupled receptors (GPRC5B and GPRC5C). Genomics 2000;67:8-18.
32 Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Soria E: Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol 2005;60:174-182.
33 Pourcel C, Salvignol G, Vergnaud G: CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology 2005;151:653-663.
34 Chalmers DT, Behan DP: The use of constitutively active GPCRs in drug discovery and functional genomics. Nat Rev Drug Discov 2002;1:599-608.
35 Eglen RM, Gilchrist A, Reisine T: The use of immortalized cell lines in GPCR screening: The good, bad and ugly. Combinatorial Chemistry & High Throughput Screening 2008;11:560-565.
36 Gurzov EN, Ortis F, Cunha DA, Gosset G, Li M, Cardozo AK, Eizirik DL: Signaling by IL-1beta+IFN-gamma and ER stress converge on DP5/Hrk activation: a novel mechanism for pancreatic beta-cell apoptosis. Cell Death Differ 2009;16:1539-1550.
37 Tessem JS, Moss LG, Chao LC, Arlotto M, Lu D, Jensen MV, Stephens SB, Tontonoz P, Hohmeier HE, Newgard CB: Nkx6.1 regulates islet beta-cell proliferation via Nr4a1 and Nr4a3 nuclear receptors. Proc Natl Acad Sci U S A 2014;111:5242-5247.
38 Furuya F, Shimura H, Yamashita S, Endo T, Kobayashi T: Liganded thyroid hormone receptor-alpha enhances proliferation of pancreatic beta-cells. J Biol Chem 2010;285:24477-24486.
39 Lee SH, Piran R, Keinan E, Pinkerton A, Levine F: Induction of beta-cell replication by a synthetic HNF4alpha antagonist. Stem Cells 2013;31:2396-2407.